

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Michael BRISTOW *et al.*

Serial No.: 09/415,733

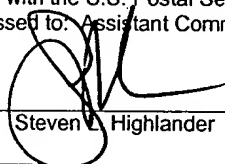
Filed: October 12, 1999

For: DIAGNOSIS AND TREATMENT OF  
MYOCARDIAL FAILURE

Group Art Unit: 1643

Examiner: J. Souaya

Atty. Dkt. No.: MYOG:004USC1/HYL

CERTIFICATE OF MAILING 37 C.F.R. § 1.8	
I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on the date below:	
April 3, 2001 Date	 Steven V. Highlander

DECLARATION OF RICK GORCZYNSKI UNDER 37 C.F.R. §1.132

Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

I, Rick Gorczynski, do declare the following:

1. I am currently hold the position of Vice President, Research & Development at Myogen, Inc., licensee of the above-captioned application. My education and training includes an undergraduate degree in Biological Sciences from Cornell University and a Ph.D in Cardiovascular Physiology from the University of Virginia, School of Medicine. I have worked since 1976 in the pharmaceutical industry, primarily in the cardiovascular drug discovery field. During my 25 years in the industry I have conducted and/or supervised research directed at a variety of cardiovascular diseases including heart failure (acute and

TNT  
considered  
11/20/01

chronic), myocardial infarction, cardiac dysrhythmia, hypertension, renal disease, hyperlipidemias and thrombosis disorders. For the past two years I have been exclusively engaged in the discovery and validation of molecular drug targets for use in drug discovery in the field of heart failure. I am intimately familiar with the myosin heavy chain and the role it plays in cardiac hypertrophy and heart failure. An updated copy of my Curriculum Vitae is attached.

2. I am also familiar with the level of skill of scientists working in the field of cardiology and molecular biology as of the priority date of the referenced application. I consider one of ordinary skill in the art in this field of study to have a Ph.D. in biochemistry, chemistry, molecular biology, pathology or other related field, or an M.D., with 1-3 years of post-graduate study.
3. I have reviewed the specification and pending claims 20-25 for the above-referenced case. Claims 21-25 depend from claim 20 which reads "A method of treating myocardial failure in a human comprising administering an effective amount of an agent that directly causes an increase in the quantity of  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) in the myocardial tissue of the heart." Claims 21-25 further specify the use of thyroid hormone, or analogs thereof, as the agent, and the specific location and nature of the myocardial tissue to be treated. The specification discloses that a correlation exists between levels of  $\alpha$ - and  $\beta$ -MHC in the heart and myocardial failure. Particularly,  $\alpha$ -MHC levels are decreased and  $\beta$ -MHC levels are increased in diseased hearts undergoing myocardial failure. Treatment of such failure by administering a therapeutic compound which increases the level of  $\alpha$ -MHC in heart tissue also is described.

4. I also have reviewed the attached scientific publication entitled "Changes in Gene Expression Associated with Phenotypic Improvement of Dilated Cardiomyopathy." Based on the results set forth in this manuscript, it is clear to me that a person of ordinary skill (as defined above) would recognize the correlation between the levels of  $\alpha$ - and  $\beta$ -MHC in myocardial tissue and myocardial failure.

The study takes advantage of the fact that  $\beta$ -adrenergic blocking agents, such as metoprolol and carvedilol, effectively improve systolic function and reverse the dilated cardiomyopathy phenotype. Thus, monitoring gene expression levels as a correlate of improvement in the phenotype provides valuable evidence regarding a relationship between expression of various genes and the disease state itself. The referenced study measured expression levels of six different genes in intact myocardial tissue as a function of left ventricular ejection fraction (LVEF), which is an effective measure of systolic contractile dysfunction. The LVEF in a group of 45 subjects (age range of  $54.1 \pm 10.5$  yrs) who exhibited the idiopathic dilated cardiomyopathy phenotype was modified by administering  $\beta$ -adrenergic blocking agents. Before administration of the  $\beta$ -adrenergic blocking agents, baseline measurements of the gene expression levels in the 45 subjects were taken and compared to the levels in 8 control subjects (age range of  $49.1 \pm 4.6$  yrs) who showed no left ventricular dysfunction. Following a six month period of administration of either a placebo (given to 13 subjects), metoprolol (given to 14 subjects) or carvedilol (given to 18 subjects), LVEF was again measured along with the levels of gene expression.

The baseline measurements showed that, when compared to the control group, the subjects with failing hearts had lower levels of several gene products including  $\alpha$ -MHC, and increased levels of several gene products including  $\beta$ -MHC. Following the six month treatment period, the 45 subjects were divided into three groups: (1) those who showed a decline or no change in LVEF; (2) those who showed an improvement in LVEF; and (3) those who showed a marked improvement in LVEF. Results of gene expression levels in each group showed a correlation between levels of  $\alpha$ - and  $\beta$ -MHC and LVEF. Linear regression analysis, with LVEF as the continuous variable, clearly shows a positive correlation with  $\alpha$ -MHC and a negative correlation with  $\beta$ -MHC.

Reduced LVEF is one of the primary variables that define heart failure severity and improvements in LVEF signal an improvement in myocardial performance in heart failure patients. Because this study is able to isolate LVEF as a variable, the results show that levels of  $\alpha$ - and  $\beta$ -MHC in myocardial tissue are correlative with myocardial failure and recovery. As the dilated cardiomyopathy phenotype is improved, the levels of  $\alpha$ - and  $\beta$ -MHC change, irrespective of the age of the subjects. Thus, even if age does contribute to  $\alpha$ - and  $\beta$ -MHC changes, it cannot be responsible for the changes observed here.

Furthermore, the significance of this study being performed using intact human heart tissue should be emphasized. Measuring gene expression levels from an intact heart helps eliminate many ancillary factors (such as homeostatic disruption of brain death) that may otherwise contribute to changes in gene expression levels. Using tissue from an intact heart also allows longitudinal studies, which, as demonstrated in the present study, allow for a more direct detection of changes in gene expression that occur as

the phenotype is modified. In addition, longitudinal studies permits use of each tissue donor as their own control, thus reducing variability. Finally, due to the combinatorial nature of gene expression, using an intact heart allows one to measure specific gene expression levels without negating the net effects of multiple regulatory influences.

5. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

April 2, 2001  
Date

Richard Gorczynski  
Richard Gorczynski, Ph.D.

# CURRICULUM VITAE

**RICHARD J. GORCZYNSKI, Ph.D.**

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Boulder, CO 80301

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## PROFESSIONAL EXPERIENCE

**12/98-present**

**Vice President, Research and Development, Myogen Inc, Aurora, Colorado**

### Responsibilities

- Corporate Officer and member of the Myogen Management Team
- Lead R&D activities focused on clinical development of a Phase III stage therapeutic for treatment of ultra-advanced heart failure; use of genomic, proteomic and biological techniques for identification of drug discovery molecular targets for treatment, reversal and prevention of heart failure; validation of molecular targets for drug discovery; identification of novel diagnostic markers for cardiac hypertrophy and heart failure
- Project Team Leader for Enoximone P.O. Development
- Project Team Leader for Myosin Heavy Chain Project (collaboration between Myogen and a big pharma company)

### Accomplishments

- In collaboration with Myogen scientific and medical advisory boards, established the company's Research Plan
- Project Plans for two Projects established: enoximone P.O. and Myosin Heavy Chain
- Planned and completed two successful meetings with the FDA Cardiorenal Division; resulted in agreement to proceed to Phase III with a heart failure therapeutic (enoximone); achieved alignment with the Agency on endpoints for four Phase II studies, product labelling language and scope of NDA.

**6/98-11/98**

**Vice President, Research and Development and Boulder-Site Manager, Baxter, Hemoglobin Therapeutics Division, (Post-Baxter acquisition of Somatogen), Boulder, Colorado**

### Responsibilities

- Member of the Hemoglobin Therapeutics Division Management Team
- Lead Research and development activities at the Boulder-Site focused on the biological support of a Phase III-stage hemoglobin product candidate, DCLHb, research and development support for a Phase II-stage hemoglobin product candidate, rHb1.1 and research and development activities related to the discovery and advancement to clinical evaluation of a Second Generation hemoglobin product candidate
- Manage the Boulder-Site administratively, including facilities, safety, MIS and communications for R&D, Operations, Clinical, HR and Finance.

Accomplishments

- Defined new Boulder-Site organization in collaboration with the hemoglobin Therapeutics Division management team
- Identified several novel Second Generation hemoglobin product candidates

**12/94 -6/98****Vice President, Research and Development, Somatogen, Inc., Boulder, CO**Responsibilities

- Corporate Officer and member of the Executive Management Group.
- Lead Research and Development activities at Somatogen; focused on 1) commercialization of lead product, Recombinant Human Hemoglobin (rHb 1.1) for oxygen-delivering and hematopoietic therapeutic indications 2) discovery and development of Second Generation recombinant hemoglobin products 3) development of non-hemoglobin technologies.
- Manage and coordinate Departments of Molecular Biology, Protein Engineering, Hemoglobin Research/Protein Chemistry, Pharmacology/Toxicology, Molecular Computation, Analytical Development, Purification Development, Formulation Development, and Fermentation Development (total of 65-70 people).

R&D Accomplishments

- In conjunction with the other Corporate Officers, positioned Somatogen for acquisition by Baxter Healthcare and prepared and presented the key technology summaries which lured Baxter to the table and eventually lead to an acquisition of Somatogen.
- Developed bioprocess for making clinical grade rHb1.1 with successful scale-up demonstrating achievement of commercial expression and downstream yield targets; includes construction of host vector and strain, fermentation process, recovery and downstream purification system and associated analytical characterization; some of this was accomplished in collaboration with Eli Lilly and Co., our strategic corporate partner at that time.
- Advanced the understanding of hemoglobin biological effects including efficacy (oxygen delivery to tissue and potency), and safety related biologic effects; this work completed in support of commercial development of rHb1.1 and extended to the discovery of novel hemoglobin products.
- Initiated research on a promising new indication for rHb1.1: tumor radiation therapy sensitization.
- Initiated a drug-discovery project to identify new generation recombinant hemoglobin with enhanced therapeutic attributes; over 600 variant recombinant and chemically modified/conjugated/cross-linked molecules constructed in three years; several lead molecules are undergoing advanced biological evaluation to ascertain suitability for human clinical testing. All have significantly improved properties.
- Three/four fold increases in rHb expression levels have been achieved (relative to commercial targets for rHb1.1).
- Completed several studies investigating the level of hematopoietic activity of rHb1.1 and other recombinant hemoglobins.
- Supervised preclinical discovery and development of a novel, in-licensed platelet substitute.

**4/93 -12/94**

**Senior Director, Drug Discovery, Searle, Skokie, IL**

Responsibilities

- Supervise the Cardiovascular Discovery Research Department (approximately 50 scientists; two sites: Skokie and St. Louis) with primary emphasis on atherosclerosis, thrombosis, arrhythmia, congestive heart failure and hyperlipidemia.
- Coordinate the process by which compounds from the Discovery Department are selected for, and transferred into, the Development Pipeline.
- Member R/D Executive Committee, Research Executive Committee and Development Executive Committee.
- Skokie Discovery Site Manager for facilities, safety and space administration.
- R/D liaison to the Corporate Licensing group.

Accomplishments

- Department Charter and Long Range Research Plan established.
- Five new Ph.D. hires in 1993 with backgrounds representing new directions in cardiovascular research (atherosclerosis/thrombosis/diabetes).
- Directed the design of a process by which Searle R/D will select Discovery stage compounds for formal Development; process consists of early toxicity, formulation, pharmacokinetic and chemical development studies of candidate molecules to optimize selection and the completion of critical analysis (development plan, marketing and financial) to support informal discussions on what to develop.
- Advanced new antiplatelet and antithrombotic agents into development (7/94); two antiplatelet compounds advanced to Phase III clinical development

**8/89 - 4/93**

**Senior Director, Scientific and Product Affairs, Licensing/Business Development, Searle, Skokie, IL**

Responsibilities

- Identification and follow-up of license and business development opportunities, with particular emphasis on Japan. Technical evaluation of all product license candidates.
- Manage process for full technical, medical and marketing review of candidates.
- Coordinate design of Development and Commercialization Plans for in-license candidates.
- Presentation of licensing opportunities to Searle top-management.
- Liaison between Licensing and Searle R/D.

Accomplishments:

- Two development collaborations initiated.
- One compound in-licensed (antidiabetic).

**1/86 - 8/89**

**Director, Department of Cardiovascular Diseases Research, Searle, Skokie, IL**

Responsibilities

- Supervise product discovery, chemical and biological research in the cardiovascular field with primary emphasis on hypertension, atherosclerosis, thrombosis and arrhythmia (staff: 45).

Accomplishments

- Four compounds into development (antihypertensive, and three antiplatelet agents).
- Two compounds in clinical study: antiarrhythmic and hypolipidemic.

**9/85 - 1/86**

**Director, Biological Research Department, Searle, Skokie, IL**

Responsibilities

- Supervised product discovery, biological research in four areas: cardiovascular, CNS, gastrointestinal and autotoxin mediated diseases (staff: 80).
- Department was reorganized 1/86 following restructuring of all R/D after Monsanto takeover of Searle.

**8/83 - 9/85**

**Section Head Pharmacology, American Critical Care** (Division of Baxter Travenol Corp) (formerly Arnar-Stone Laboratories), McGaw Park, IL

Responsibilities

- Supervised drug discovery, biological research in the cardiovascular, ophthalmic and CNS areas including beta-blockers, positive inotropic agents, antiarrhythmic agents, antiglaucoma agents and antiepileptic agents (staff: 13).

Accomplishments

- Five compounds into development (two beta-blockers, one antiglaucoma, one antiarrhythmic and one antiepileptic).
- Four IND's and one NDA (with approval).

**9/80 - 8/83**

**Group Leader, American Critical Care** (Division of American Hospital Supply Corp.) McGaw Park, IL,

Responsibilities

- Supervised drug discovery, biological research in the cardiovascular area (beta-blockers, cardiotonics and alpha blockers).

**11/78 - 9/80**

**Senior Research Investigator, Arnar-Stone Laboratories** (Division of American Hospital Supply Corp.), McGaw Park, IL,

Responsibilities

- Drug discovery in field of dopamine analogues and beta adrenergic receptor antagonists.

**9/76 - 11/78**

**Research Investigator, Arnar-Stone Laboratories, McGaw Park, IL****Responsibilities**

- Drug discovery in field of dopamine analogues and beta adrenergic receptor antagonists.

**DRUG DEVELOPMENT EXPERIENCE**

- Project Team Leader: enoximone P.O.; Phase II for treatment of ultra-advanced heart failure (myogen).
- Project Team Leader: Second Generation recombinant hemoglobin project (Somatogen).
- Designed the process used to select compounds for formal Development and Clinical Study (Searle).
- Liaison to Development Project Teams for all cardiovascular compounds accepted for development (Searle).
- Project Team Leader (American Critical Care).
- Coordinate design of Development and Commercialization Plans for in-license candidates.
- Development of an ultra-short acting beta-blocker - responsible for organizing and tracking development of a novel compound through all stages of preclinical development (raw material supplies, pharmacology, drug metabolism/pharmacokinetics, analytical assays, formulation, stability, etc.) and initial clinical trials.
- Member of three other project teams which are responsible for the development of a vasodilator, an antiarrhythmic agent and another ultra-short acting beta-blocker.

**EDUCATION**

1976 Ph.D., Physiology  
University of Virginia  
School of Medicine, Department of Physiology  
Charlottesville, Virginia

Dissertation: The Microcirculatory Basis of Functional  
Hyperemia in Striated Muscle (University Microfilms #76-25012)

Thesis

Advisor: Brian R. Duling, Ph.D., Professor of Physiology

1970 B.A., Biological Sciences  
Cornell University  
Ithaca, New York

**TRAINING**

- 1996 Somatogen: Performance Management System
- 1995 Somatogen: Project Management
- 1990 Searle: Introduction to Financial Analysis in Business (AMA)
- 1989 Searle: Introduction to Licensing (LES)
- 1989 Searle: Decision Making Skills: Consensus
- 1986 Searle: Interview Selection Skills
- 1986 Searle: Personnel Management System Training
- 1983 American Hospital Supply: Corporate Middle Management Course
- 1981 American Management Association: Project Management
- 1980 American Hospital Supply: Management Style and Effectiveness Training

### **AWARDS**

American Critical Care President's Award for Scientific and Technical Excellence - 1979

Runner-up for American Critical Care President's Award for Scientific and Technical Excellence - 1978

### **PROFESSIONAL ACTIVITIES**

Member, Editorial Board of the Journal of Cardiovascular Pharmacology - 1984 to 1994

Ad Hoc Reviewer for Microvascular Research, the American Journal of Physiology and the Journal of Pharmacology and Experimental Therapeutics, Blood

### **SOCIETIES**

International Society for Artificial Cells, Blood Substitutes and Immobilization Biotechnology (Scientific Steering Committee)

American Society for Pharmacology and Experimental Therapeutics  
American Association for Advancement of Science  
International Society for Heart Research  
Licensing Executives Society  
American Heart Association

### PATENTS

Novel therapeutic and diagnostic agents for treatment of heart failure. Applied May, 1999.

Epoxy-Steroidal Aldosterone Antagonist and Angiotensin II Antagonist Combination Therapy for Treatment of Congestive Heart Failure. WO96/40257

### SEMINARS

1. Department of Physiology, University of Virginia, Fall 1976. "The microcirculatory basis of functional hyperemia in striated muscle".
2. Department of Physiology, Medical College of Wisconsin, Fall 1977. "The microcirculatory basis of functional hyperemia in hamster striated muscle".
3. Cardiovascular Discussion Group, Skokie, IL, Fall 1982. "Mechanisms of inotropic selectivity".
4. Esmolol Symposium, Spring 1985. "Basic pharmacology of esmolol".
5. Kureha Chemical Industry, Tokyo, Fall 1989. "Platelet GPIIb/IIIa: a new target for discovery of novel antiplatelet agents".
6. University of Virginia, Graduate Study Colloquium, Winter, 1994. "Job Opportunities in the Pharmaceutical Industry."
7. IBC Conference Blood Substitute, 1996. "Measurement of the Efficacy of Hemoglobin-based Oxygen Carriers
8. International Symposium on Intensive Care and Emergency Medicine, Brussels, 1997. Preclinical update on rHb1.1
9. Tokyo Blood Substitutes Conference, 1997. "Comparison of Optro with Whole Blood using  $^{31}\text{P}$ -NMR Spectroscopy"

## PUBLICATIONS

1. Spath, J.A., Gorczynski, R.J. and Lefer, A.M.: Possible mechanisms of the beneficial action of glucocorticoids in circulatory shock. Surg. Gyne. and Obst., 137:597-607, 1973
2. Spath, J.A., Gorczynski, R.J. and Lefer, A.M.: Pancreatic perfusion in the pathophysiology of hemorrhagic shock. Amer. J. Physiol., 226:443-451, 1974
3. Gorczynski, R. J., Spath, J.A. and Lefer, A.M.: Vascular responsiveness of the in situ perfused dog pancreas. Europ. J. Pharmacol., 27:68-77, 1974
4. Gorczynski, R.J. and Lefer, A.M.: Properties of the reticuloendothelial system of the cat. Proceed. Soc. Exper. Biol. & Med., 147:24-28, 1974
5. Gorczynski, R.J., Klitzman, B.M. and Duling, B.R.: Interrelations between contracting striated muscle and precapillary microvessels. Amer. J. Physiol., 235:H494-H504, 1978
6. Gorczynski, R.J. and Duling, B.R.: The role of oxygen in arteriolar functional vasodilation in hamster striated muscle. Amer. J. Physiol., 235:H505-H515, 1978
7. Borgman, R.J., Erhardt, P.W., Gorczynski, R.J. and Anderson, W.G.: Cyclopropylamine hydrochloride (ASL-7003): A rigid analogy of dopamine. J. Pharm. Pharmacol., 30:193-195, 1978
8. Gorczynski, R.J., Anderson, W.G., Erhardt, P.W. and Stout, D.M.: Analysis of the cardiac stimulant properties of (3,4-dihydroxyphenyl)-cyclopropylamine (ASL-7003) and 2-Amino-6,7-Dihydroxy-1,2,3,4-Tetrahydronaphthalene (A6,7DTN). J. Pharm. Exp. Therap., 210(2):252-258, 1979
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10. Erhardt, P.W., Gorczynski, R.J. and Anderson, W.G.: Conformational analogues of dopamine. Synthesis and pharmacological activity of (E)- and (Z)-2-(3,4 dihydroxyphenyl) cyclopropylamine hydrochlorides. J. Med. Chem., 22 (8):907-911, 1979
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12. Reynolds, R.D., Burmeister, W.E., Gorczynski, R.J., Dickerson, D.D., Mathews, M.P. and Lee, R.J.: Effects of propranolol on myocardial infarct size with and without coronary artery reperfusion in the dog. Cardiovas. Res., 15 (8):411-420, 1981
13. Gorczynski, R.J., Anderson, W.G. and Stout, D.M.: N-aralkyl substitution of 2-amino-5,6-and -6,7-dihydroxy-1,2,3,4-tetrahydronaphthalenes. 1. Cardiac and pressor/depressor activities. J. Med. Chem., 24:835-839, 1981
14. Stout, D.M. and Gorczynski, R.J.: N-aralkyl substitution of 2-amino-5,6- and -6,7,8-dihydroxy-1,2,3,4-tetrahydronaphthalenes. 2. Derivatives of a hypotensive-positive inotropic agent. J. Med. Chem., 25:326-328, 1982
15. Gorczynski, R.J.: Cardiovascular pharmacology of ASL-7022, a novel catecholamine. I. Inotropic, chronotropic and pressor actions. J. Pharm. Exp. Therap., 223 (1): 7-11, 1982
16. Gorczynski, R.J. and Wroble, R.W.: Cardiovascular pharmacology of ASL-7022. II. Mechanisms of inotropic selectivity. J. Pharm. Exp. Therap., 223 (1):12-19, 1982
17. Zaroslinski, J., Borgman, R.J., O'Donnell, J.P., Anderson, W.G., Erhardt, P.W., Kam, S-T, Reynolds, R.D., Lee, R. J. and Gorczynski, R.J.: Ultra-short acting beta-blockers: A proposal for the treatment of the critically ill patient. Life Sciences, 31:899-907, 1982
18. Erhardt, P.W., Woo, C.M., Gorczynski, R.J. and Anderson, W.G.: Ultra-short-acting beta-adrenergic receptor blocking agents. 1. (Aryloxy)propanolamines containing esters in the nitrogen substituent. J. Med. Chem., 25:1402-1407, 1982
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20. Klitzman, B., Damon, D.N., Gorczynski, R.J. and Duling, B.R.: Augmented tissue oxygen supply during striated muscle contraction in the hamster: Relative contributions of capillary recruitment, functional dilation and reduced tissue PO<sub>2</sub>. Circulation Research, 51:711, 1982
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27. Kam, S-T., Matier, W.L., Mai, K.X., Anderson, W.G., Gorczynski, R.J. and Lee, R.J.: [(Arylcarbonyl)oxy]propanolamines: New catechol beta-blockers with little beta-intrinsic activity. J. Med. Chem., Sept., 1983
28. Gorczynski, R.J., Murthy, V.S. and Hwang, T.F.: Beta-blocking and hemodynamic effects of ASL-8052. J. Cardiovas. Pharm., 6:1048-1059, 1984
29. Shaffer, J.E. and Gorczynski, R.J.: Role of alpha-adrenergic receptors in the intrinsic inotropic selectivity of dobutamine in anesthetized dogs. Canad. J. Physiol. Pharm., 63 (6):630-635, 1985
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# Changes in Gene Expression Associated with Phenotypic Improvement of Dilated Cardiomyopathy

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## ABSTRACT

We tested the hypothesis that dynamic changes in the expression of specific contractility regulating genes would selectively accompany phenotypic modification of idiopathic dilated cardiomyopathy (IDC) in human subjects with chronic heart failure. Expression of contractility- and pathologic hypertrophy-associated genes was measured in total RNA extracted from milligram amounts of endomyocardial biopsy material, by reverse transcription-quantitative polymerase chain reaction. Gene expression, myocardial  $\beta$ -adrenergic receptor protein density and ventricular function determined by radionuclide ejection fraction were assessed in 45 subjects with chronic heart failure from IDC, at baseline and after 6 months of treatment with either  $\beta$ -adrenergic blocking ( $n=32$ ) agents or placebo ( $n=13$ ). At baseline IDC ventricles exhibited induction of the "fetal" gene program and down-regulation of  $\beta_1$ -adrenergic receptor gene and protein expression. Based on behavior of left ventricular ejection fraction (LVEF) over the 6 month treatment period the 45 IDC subjects were rank-ordered into tertiles. This created a group of 15 subjects who exhibited a decline or no change in LVEF (mean of  $-2.2 \pm 1.1$  (SEM) EF units), 15 with improved function (mean of  $+12.2 \pm 0.9$ ), and 15 with markedly improved function (mean of  $+25.5 \pm 1.8$ ). Changes in the expression of three sets of contractility-regulating genes ( $\beta$ -adrenergic receptors ( $\beta$ -ARs), sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SRCA) and  $\alpha$ - and  $\beta$ -myosin heavy chain (MyHC) isoforms), as well as a pathologic hypertrophy molecular marker (atrial natriuretic peptide, ANP) were compared across the LV function change groups. As LVEF and RVEF improved, the steady state abundance of  $\alpha$ -MyHC mRNA increased and  $\beta$ -MyHC mRNA decreased. There were no statistically significant changes in  $\beta_1$  or  $\beta_2$ -adrenergic receptor mRNA or protein expression, or in SRCA or ANP mRNA expression across ventricular function change groups. We conclude that in human dilated cardiomyopathy phenotypic modification is selectively associated with changes in MyHC isoforms in a manner that would improve contractile function and reduce pathologic hypertrophy.

## INTRODUCTION

Disease phenotypes are ultimately the result of changes in gene expression. In the chronically failing, hypertrophied human heart selective changes in the expression of genes that could potentially modify phenotype have been reported, most commonly in explanted human hearts at the end-stage of the disease process.<sup>1-12</sup> Reported alterations in gene expression in this setting include changes in the mRNA or protein expression of components of  $\beta$ -adrenergic signal transduction,<sup>2-5</sup> calcium handling proteins<sup>6-8</sup> and contractile proteins.<sup>9-11</sup> However, in these studies it has been difficult to determine which alterations in gene expression are fundamentally related to contractile dysfunction as opposed to those which are epi-phenomena associated with advanced disease. Another interpretative challenge in these studies has been the difficulty of distinguishing between adaptive vs. maladaptive processes, since many of the described changes are products of compensatory mechanisms that can produce either benefit or harm.

Additionally, because end-stage failing hearts or the organ donor-procured controls used in these studies may be affected by a multitude of ancillary factors it is important to ultimately investigate gene expression in the intact heart. In the intact heart starting material can be obtained from less advanced myocardial failure, and controls are not subjected to the profound homeostatic disruption of brain death.<sup>12,13</sup> Also, studies in the intact heart may be conducted longitudinally,<sup>14-16</sup> which would theoretically allow for detection of gene expression changes more directly associated with phenotypic modification. Importantly, because the regulation of gene expression is combinatorial,<sup>17</sup> a strong case can be made for investigating gene expression in intact tissues where the net effect of multiple regulatory influences can be assessed.

Using reverse transcription-quantitative PCR (RT-QPCR) it is possible to measure the steady-state mRNA abundance of multiple genes in small amounts of starting material,<sup>18</sup> such as endomyocardial biopsy.<sup>1,9,16</sup> Treatment of chronic heart failure subjects with  $\beta$ -adrenergic blocking agents is associated with improvement in systolic function and a reversal of remodeling in the majority of subjects with a dilated phenotype,<sup>19</sup> within a time-frame of 4-12 months<sup>20-25</sup> that is amenable to serial investigation. This contrasts with the natural history of dilated

cardiomyopathies, which is that of slow (time course of years) progression in phenotype and clinical sequelae.<sup>26-29</sup> The current investigation reports changes in the expression of contractility-regulating genes that are associated with modifications in structure and function in the intact human heart, in a subject population of idiopathic dilated cardiomyopathy treated for 6 months with placebo or  $\beta$ -adrenergic blockade.

## METHODS

### Clinical protocol

Subjects of either sex between 18 and 80 years of age who had chronic (> 6 months duration of symptoms) symptomatic heart failure from idiopathic dilated cardiomyopathy (IDC) were eligible for enrollment in the clinical protocol. Additionally, subjects had to have an indication for a baseline endomyocardial biopsy, which at our two institutions is routinely done in IDC to rule out inflammatory or infiltrative processes. The baseline left ventricular ejection fraction had to be  $\leq .35$  measured by radionuclide ventriculography (RVG), with an increased LV size as assessed by 2-D echocardiography. Mandatory background therapy was an ACE inhibitor and digoxin, plus diuretics as needed.

Eligible subjects then signed written, informed consent approved by the Institutional Review Boards (IRBs) of the University of Colorado or Utah Health Sciences Centers, and received a battery of baseline tests designed to measure myocardial gene expression, left and right ventricular function and cardiac adrenergic drive. Following completion of baseline tests subjects were randomly assigned to three types of treatment: placebo; metoprolol or carvedilol. To maintain a double-blind carvedilol, metoprolol or placebo tablets were pulverized and placed in gelatin capsules by the research pharmacists at the respective institutions. The initial dose of carvedilol was 3.125 mg b.i.d., and for metoprolol 6.25 mg b.i.d. All subjects tolerated initiation of study medication. The dose of study medication was doubled every week until weight adjusted target doses were reached (subjects  $\geq 85$  Kg, 50 mg b.i.d. for carvedilol and 100 mg

b.i.d. for metoprolol; < 85 Kg, 25 mg b.i.d. for carvedilol and 50 mg b.i.d. for metoprolol), or until limiting treatment side effects occurred. At the end of 6 months of treatment all baseline tests were repeated. Following completion of these baseline tests all subjects had exercise tolerance and certain other measurements re-investigated after a 48 hour withdrawal period (which will be reported elsewhere), and then all subjects were converted to treatment with open-label carvedilol starting at 6.25 mg b.i.d. A diagram of this three-arm study is given in **Figure 1**.

The three-way randomization to placebo, metoprolol and carvedilol was stopped on January 13, 1998 after the estimated sample size of 42 had been reached (45 subjects enrolled) for detection of differences in  $\beta$ -receptor protein and gene expression. Because of unanticipated findings on the expression of other genes that were approaching significance in subjects with improved LV function in one or both  $\beta$ -blocker groups<sup>30</sup> the trial continued as a two-way randomization between metoprolol and carvedilol with the enrollment of an additional 9 subjects, for a total of 54. One of the subjects enrolled in the three-arm phase of the protocol was dropped from the study prior to randomization because the baseline radionuclide ejection fraction returned at > 35%. Of the 53 subjects who received complete baseline studies, 15 were randomized to placebo, 17 to metoprolol and 21 to carvedilol. All 15 placebo-treated subjects, 14 of 17 metoprolol-treated subjects and 20 of 21 carvedilol-treated subjects finished the 6 month treatment period. Of the 3 metoprolol-treated subjects who did not finish the study, one underwent cardiac transplantation enrollment, and two dropped out. The carvedilol-treated subject who did not complete the study died suddenly 6 weeks after enrollment. Of the 49 subjects who completed the study, complete sets of gene expression measurements were performed at baseline and end of study on 45, who form the basis of this report. Thirteen of these subjects were treated with placebo, 14 with metoprolol and 18 with carvedilol. The mean  $\pm$  SD daily total dose of metoprolol used in these subjects was  $125 \pm 47$  mg with a range of 50-200 mg, and for carvedilol it was  $70 \pm 29$  mg with a range of 25-100 mg.

In addition, in 35 of the 45 subjects enough endomyocardial biopsy material (an additional  $\geq 10$ mg) was available to measure  $\beta$ -receptor protein density. There were no complications of

endomyocardial biopsy or right heart catheterization. No infiltrative or inflammatory processes were identified by light microscopy, which revealed evidence of hypertrophy and variable degrees of interstitial fibrosis in all cases.

Finally, baseline  $\beta$ -adrenergic receptor, gene expression and myocardial functional data in the IDC subjects was compared to data generated in 8 subjects without LV dysfunction, who were biopsied as part of a separate IRB-approved protocol conducted at the University of Colorado to compare  $\beta$ -adrenergic receptor and gene expression profiles across various types of nonfailing and failing hearts. All subjects with nonfailing LVs had a clinical indication for endomyocardial biopsy: three had possible cardiac symptoms and were referred for a clinical suspicion of a myocardial disease process, and five had treatment protocol-specified biopsies performed prior to enrollment in chemotherapy protocols that included agents that were potentially cardiotoxic. All subjects with normal LV function signed written informed consent for investigation of gene expression and receptor profiles across patient populations.

#### **Right ventricular endomyocardial biopsy and right heart catheterization**

Right heart catheterization was performed from the right internal jugular (RIJ) vein as previously described.<sup>9,14,15</sup> Following cannulation of the RIJ, endomyocardial biopsy of the distal RV septum was performed with a Mansfield "Large Bite" bioptome under both fluoroscopic and echocardiographic guidance to ensure proper positioning of the biopsy forceps. Six to eight samples of endomyocardium weighing 25-30 mg were taken, and allocated to  $\beta$ -adrenergic receptor measurements (10 mg), gene expression measurements (10 mg) and routine light microscopy. Following endomyocardial biopsy, hemodynamics were measured using a balloon-tipped catheter.

#### **Gene expression measurements in endomyocardial biopsy material**

Total RNA was extracted from 2-4 endomyocardial biopsies (a total of 4-8 mg) by the guanididum thiocyanate phenol-chloroform method using RNA STAT-60, as previously

described.<sup>9</sup> A double extraction was routinely used to eliminate small amounts of DNA contamination. Messenger RNA abundance was measured by reverse transcription-quantitative PCR (RT-QPCR) according to previously described methods.<sup>1,9</sup> This RT-QPCR method is based on simultaneous reverse transcription and PCR amplification of a known amount of internal standard cRNA of slightly smaller size than the PCR product generated from the transcript of interest.<sup>1,9</sup> The internal standard and the cDNA produced from the transcript of interest are then collinearly amplified in the same reaction tube, and the PCR products are quantified by using <sup>32</sup>P-end-labeled primers. From regions of the two curves that collinearly amplify it is then possible to determine the original amount of unknown mRNA, provided that a known amount of internal standard is added. This method yields an absolute measurement of mRNA abundance, in molecules x 10<sup>5</sup>/μg total RNA, with total RNA being directly measured by a small volume, high sensitivity spectrophotometer.<sup>1,9</sup> Using this form of RT-QPCR it is possible to measure the expression of multiple genes of interest in small amounts of human ventricular myocardium; the current protocol measured the expression of six different genes (β<sub>1</sub>- and β<sub>2</sub>-adrenergic receptors, α- and β-myosin heavy chain (MyHC), atrial natriuretic peptide (ANP), and SR Ca<sup>2+</sup> ATPase (SERCA-2a or SRCA).

**β-adrenergic receptor measurements in endomyocardial biopsy material, catecholamine measurements.**

Total β receptor density and β<sub>1</sub>- and β<sub>2</sub>-receptor subtypes were measured in biopsy material as previously described.<sup>9</sup> Norepinephrine and epinephrine were determined in plasma from coronary sinus blood by a previously described radioenzymatic method.<sup>15</sup>

**LV, RV ejection fraction measurements by radionuclide ventriculography**

Left and right ventricular ejection fractions were measured by previously described in vitro red blood cell labeling and imaging techniques,<sup>22,24</sup> and the results were expressed in EF units ([stroke volume/end diastolic volume] x 100). Briefly, in both institutions gated blood pool

imaging was performed using a small field of view scintillation camera which acquired 16 frames per cardiac cycle and 250K counts/frame into a 64 X 64 matrix to obtain greater than 2 million counts for the entire study. R-wave gating was used with a standard 15% R-R window and a double buffered "bad beat" rejection algorithm. Right and left ventricular ejection fractions (EFs) were computed using a manually corrected semi-automated analysis computer method, with the operator blinded to the research subject's treatment assignment. For RVEF a first pass technique was employed, and for LVEF an equilibrium technique was used.

### Statistical analysis

The 45 subjects who had baseline and completion of study gene expression measurements were rank ordered by change in left ventricular ejection fraction (LVEF) into 3 equal groups in ascending order of LVEF change, regardless of treatment type. Thus 15 subjects who had an LVEF change between -11 and +4 (average  $-2.2 \pm 1.1$ (SEM)) EF units comprised the *declined/no change* function group, 15 subjects who had an LVEF change between +5 and +17 (average  $12.2 \pm 0.9$ ) EF units comprised the *improvement* group, and 15 subjects with changes between +18 and +44 (average  $25.5 \pm 1.8$ ) EF units comprised the *marked improvement* group. We chose to rank order ventricular function change by LVEF rather than RVEF because 1) changes on the right side of the septum reflect processes occurring in both the left and right ventricles, since the septum is a wall shared by both chambers,<sup>31,32</sup> 2) with radionuclide techniques LVEF is more accurately measured than is RVEF,<sup>33,34</sup> 3) 14 of the 45 study subjects had normal ( $\geq 40$  EF units) RVEF values at baseline, and 4) as shown in **Results**, the ordered classification generated by LVEF changes also resulted in incrementally increasing RVEF changes.

Gene expression and receptor protein changes among the three LV function change groups were then analyzed by ANOVA and the Scheffe multiple comparison test, as were differences among treatment groups. The relationship of changes in LV function to changes in gene expression was also assessed by linear regression analysis treating LVEF change as a continuous

variable. LVEF or RVEF differences among treatment groups were assessed by contingency table analysis of the rank-ordered EF changes into Decline/no change, Improved and marked Improvement groups. Finally, differences within functional change and treatment groups were assessed by Student's paired t-test.

Baseline data are given with variance estimated as standard deviation (SD), while changes are given with variance estimated as standard error of the mean (SEM). A p value < .05 in a two-tailed distribution was considered to be statistically significant.

## RESULTS

### Subject demographics

**Table 1** gives the demographics of the 45 subjects with Class III or IV heart failure from idiopathic dilated cardiomyopathy. As can be observed in **Table 1**, the subject population is a relatively young (average age 54 years) heart failure group with moderate symptoms (majority NYHA Class III), severe LV dysfunction (LVEF 21 EF Units), preserved resting hemodynamics (cardiac index 2.46 ml/min/m<sup>2</sup>), moderate exercise intolerance (peak VO<sub>2</sub> 16.0 ml/kg/min), and marked cardiac adrenergic activation (coronary sinus norepinephrine 1010 pg/ml).

### Baseline data

Baseline gene expression and  $\beta$ -receptor protein data are given in **Table 2**. Compared to endomyocardial biopsy material taken from nonfailing human hearts, the failing hearts in the current study population exhibited decreases in the mRNA expression of the  $\beta_1$ -adrenergic receptors, SR Ca<sup>2+</sup> ATPase, and  $\alpha$ -myosin heavy chain genes, and increases in the expression of the atrial natriuretic peptide and  $\beta$ -myosin heavy chain genes. For  $\beta$ -adrenergic receptor protein expression, there were decreases in  $\beta_1$ -adrenergic receptor density, total  $\beta$ -receptor density, and % of  $\beta_1$ -adrenergic receptors.

## Effect of treatment

### Among-, between-group changes

For the 3 treatment groups, the only statistically significant change among groups was in LV LVEF (ANOVA  $p = .034$ ). The change in LVEF was  $4.5 \pm 3.1$  in the placebo group,  $13.9 \pm 2.5$  in the metoprolol group, and  $15.6 \pm 3.2$  in the carvedilol group (significantly different from placebo by Scheffe F test). RVEF changes were respectively  $4.5 \pm 2.3$ ,  $9.5 \pm 3.1$  and  $8.9 \pm 3.0$  ( $p = \text{NS}$ ) in the placebo, metoprolol and carvedilol groups. By contingency table analysis of the three function change groups, the two  $\beta$ -blocker groups had greater ( $p = .021$ ) numbers of subjects in the Improved and Marked Improvement groups (**Table 3**). For LVEF, 69 % of placebo-treated subjects were in the Decline/no change function change group, compared to 14 % and 22 % respectively for metoprolol and carvedilol. In contrast, 36 % of metoprolol- and 44 % of carvedilol-treated subjects exhibited Marked Improvement, compared to 15 % of placebo-treated subjects. Contingency table analysis for RVEF change was not statistically significant.

### Changes within-groups

There were multiple significant changes ( $p < .05$ ) in the measured parameters within treatment groups. These were: placebo group, increases in total  $\beta$ - and  $\beta_1$ -adrenergic adrenergic receptor densities, and decreases in ANP mRNA abundance and coronary sinus norepinephrine; metoprolol group, increases in total  $\beta$ - and  $\beta_1$ -adrenergic receptor densities, LVEF, RVEF, stroke volume index and stroke work index, and decreases in heart rate; carvedilol group, increases in  $\beta_1$ -adrenergic receptor density, LVEF, RVEF, stroke volume index and LV stroke work index, and decrease in ANP mRNA abundance, pulmonary wedge mean pressure, pulmonary artery mean pressure and heart rate.

## **Changes in hemodynamics, cardiac adrenergic drive, functional capacity and RVEF by LV function change group or linear regression analysis of LVEF change**

### Among-, between-group changes

**Figure 2 and Table 4** gives the changes in LVEF and RVEF and hemodynamics in the 3 groups arranged for ascending increases in LVEF. As can be observed in **Table 4** and **Figure 2**,

both LVEF and RVEF data exhibit sequential increases among the 3 LV function change groups. In addition, pulmonary wedge pressure and pulmonary artery mean pressure exhibit decreases in the Marked Improvement group vs. the Improvement or Decline/no change groups.

In terms of linear regression analysis using change in LVEF as a continuous variable, RVEF change was positively related ( $r = .51$ ,  $p = .0004$ ), and pulmonary wedge mean pressure ( $r = -.32$ ,  $p = .035$ ), pulmonary artery mean pressure ( $r = -.37$ ,  $p = .014$ ) were negatively related.

#### Changes within-groups

In terms of functional or hemodynamic changes within groups, the Decline/no change group exhibited a decrease in heart rate and an increase in stroke volume index. The Improved group had increases in LVEF, RVEF and stroke volume index, and a decrease in heart rate. The Marked Improvement group exhibited an increase in LVEF, RVEF, stroke volume index and left ventricular stroke work index, and a decrease in pulmonary wedge mean pressure, pulmonary arterial mean pressure, and heart rate.

#### **Changes in mRNA expression by LV function change group or linear regression analysis of LVEF change**

##### Among-, between-group changes

**Figures 3-7** give the changes in gene and receptor protein expression in the function change groups. As can be observed in **Figure 3**, there were no changes in  $\beta$ -adrenergic receptor gene expression associated with changes in left or right ventricular function. There were also no changes in  $\beta_1$ - or  $\beta_2$ -adrenergic mRNA abundance within any of the function change groups.

**Figure 4** demonstrates that SR  $\text{Ca}^{2+}$  ATPase (SRCA) and atrial natriuretic peptide (ANP) gene expression is not different among the different functional groups. Within function change groups there was a significant reduction in ANP expression in the Improved group, and trends for decreases within the other two groups

In contrast, there were changes in both  $\alpha$ - and  $\beta$ -myosin heavy chain that incrementally increased with the function change groups (**Figure 5**). Across the 3 LV function change groups there were ascending increases in  $\alpha$ -MyHC (ANOVA  $p = .016$ ) and ascending decreases in  $\beta$ -

MyHC (ANOVA  $p = .022$ ). A similar pattern of ascending decrease was shown for total MyHC (Figure 6). Because of the progressive increases in  $\alpha$ -MyHC and decreases in  $\beta$ -MyHC gene expression with increasing LV and RV function, there was a progressive increase in the %  $\alpha$ -MyHC/total MyHC as function improved (Figure 6).

For linear regression analysis using change in LVEF as a continuous variable, LVEF change was positively related to change in  $\alpha$ -MyHC steady state abundance ( $r = .36$ ,  $p = .016$ ), and negatively related to change in  $\beta$ -MyHC ( $r = -.44$ ,  $p = .004$ ) and total MyHC ( $r = -.42$ ,  $p = .006$ ). No other changes in gene expression were significantly related to change in LVEF.

#### Changes within-groups

Within function change groups, the Declined/no change group exhibited an increase ( $p < .05$  by paired t-test) in  $\beta$ -MyHC and total MyHC, and a trend ( $p < .10$ ) for a decrease in ANP gene expression; the Improved group had an increase in  $\alpha$ -MyHC and %  $\alpha$ -MyHC plus a trend for an increase in  $\beta_2$ -receptor, and a decrease in ANP gene expression; and the Marked Improvement group exhibited trends in increases in  $\alpha$ -MyHC and %  $\alpha$ -MyHC, and trends for decreases in  $\beta$ -MyHC and total MyHC gene expression.

### **Changes in $\beta$ -adrenergic receptor protein expression by LV function change group or linear regression analysis of LVEF change**

#### Among-, between-group changes

As can be observed in Figure 7, there were no differences in the expression of  $\beta_1$ -,  $\beta_2$ - or total  $\beta$ -adrenergic receptor protein expression among the 3 functional change groups. Similarly, there were no significant differences between LV function change in receptor protein expression using delta LVEF as a continuous variable.

#### Changes within-groups

As shown in Figure 7, all groups exhibited similar within-group increases in  $\beta_1$ - and total  $\beta$ -adrenergic receptor densities, and the Improved group exhibited a trend ( $p = .054$ ) for an increase

in  $\beta_2$ -adrenergic receptor protein expression that was not present in the Decline/no change or Marked Improvement group.

## DISCUSSION

From a healthcare cost standpoint heart failure is the most important medical problem in the U.S.<sup>35</sup> The most common cause of heart failure is a secondary or primary dilated cardiomyopathy, disorders characterized by hypertrophy, ventricular chamber dilatation, and decreased contractile function.<sup>27</sup> The alterations in gene expression responsible for dilated cardiomyopathy phenotypes remain obscure. In terms of contractile dysfunction, the leading contenders are changes in components of  $\beta$ -adrenergic signal transduction, calcium handling mechanisms and contractile proteins.<sup>36</sup> For ventricular pathologic hypertrophy, a net increase in contractile protein and sarcomeres through the activation of "fetal" genes that include  $\beta$ -myosin heavy chain ( $\beta$ -MyHC) has been described.<sup>9,10,37,38</sup> The induction of pathologic hypertrophy and the development of contractile dysfunction may be related, since the increased expression of  $\beta$ -MyHC and coordinate decreased expression of  $\alpha$ -MyHC and other genes such as SR  $\text{Ca}^{2+}$  ATPase decreases systolic and diastolic function.<sup>36,37</sup>

In this study we utilized the ability of  $\beta$ -adrenergic blocking agents to reverse the dilated cardiomyopathy phenotype as a tool to investigate gene expression changes associated with favorable phenotypic modification.  $\beta$ -blocker related reversal of the dilated cardiomyopathy phenotype involves improvement in velocity of pressure development,<sup>20,21</sup> an upward and leftward shift in depressed left<sup>19-21</sup> and right<sup>24</sup> ventricular function curves indicative of improved intrinsic systolic function, and a decrease in ventricular volume<sup>19-24</sup> and mass.<sup>20,25</sup> A "primary" dilated cardiomyopathy, idiopathic dilated cardiomyopathy (IDC), was chosen for investigation because IDC is relatively common and its gene expression profile is similar to more common secondary cardiomyopathies.<sup>10,27,38</sup> The high response rate of IDC to  $\beta$ -blockade plus spontaneous improvement in some subjects in the placebo group led to substantial numbers of

subjects exhibiting improvement in LVEF, including 15 subjects with a change of > 17 EF Units, a degree of improvement that amounts to normalization or near-normalization of left ventricular function. LVEF was used to measure systolic contractile function because the test is readily available and is standardized, and because LVEF is less load dependent than standard hemodynamic measurements.<sup>39</sup> In addition, because the ejection fraction calculation is heavily influenced by end diastolic volume, in dilated cardiomyopathies LVEF is also an estimate of the degree of chamber dilatation or remodeling.<sup>40</sup>

The IDC population investigated in this study exhibited the gene expression abnormalities previously reported by our<sup>1-3,5,9</sup> and other<sup>4,6-8</sup> laboratories. Although some previous studies including our own<sup>9</sup> had not found a statistically significant decrease in SR Ca<sup>2+</sup> ATPase gene expression in failing human hearts, in the large sample size examined in this study we did find a small (by 27 %), statistically significant decrease. In the current study the previously described myocardial failure-associated decrease in  $\beta_1$ -adrenergic receptor gene expression<sup>4,5,9</sup> was accompanied by a comparable decrease in protein expression, and compared to nonfailing controls neither  $\beta_2$ -receptor gene or protein expression were altered. The gene expression changes noted in the intact failing human hearts found in this and our previous report<sup>9</sup> can be categorized as including components of fetal gene program induction (the changes in MyHC isogenes, ANP and SR Ca<sup>2+</sup> ATPase), and consequences of chronic adrenergic activation (down-regulation in  $\beta_1$ -receptor gene and protein expression). The question addressed by this study is which of these gene expression abnormalities most closely relates to modifications in dilated cardiomyopathy phenotype measured over a six month period.

The results of this study indicate that changes in the expression of two genes whose regulation is coordinately linked,<sup>40</sup>  $\alpha$ - and  $\beta$ -myosin heavy chain (MyHC), are selectively associated with improvement in ventricular systolic function and reversal in remodeling. The expression of  $\alpha$ -MyHC exhibited a statistically significant progressive increase across ascending LV function change groups, from a negative value in the Decline/no change group to increasingly positive values in the Improved and Marked Improvement groups. Conversely,  $\beta$ -

MyHC gene expression exhibited a decrease from a positive change value in the Decline/no change group to progressively negative values in the Improved and Marked Improvement groups. If translated into proportional changes in protein expression, such changes in MyHC isogene expression would be expected to improve intrinsic systolic and diastolic function.<sup>9,42,43</sup> This is because the  $\alpha$ -MyHC isoform has a much higher myosin ATPase activity than the  $\beta$ -isoform, which results in a faster velocity of shortening.<sup>42,44</sup> In rodent<sup>45,46</sup> or rabbit<sup>47</sup> models of pathologic hypertrophy and myocardial failure, coordinate decreases in  $\alpha$ -MyHC and increases in  $\beta$ -MyHC mRNA and protein expression are associated with a reduction in velocity of shortening and other measures of systolic function. Although in the current study the small amounts of starting material precluded measurement of  $\alpha$ - and  $\beta$ -MyHC protein, in gram quantities of failing explanted human left ventricular myocardium gel electrophoresis-measured MyHC isoforms exhibit changes that are directionally similar to the changes in mRNA.<sup>43</sup> Therefore, it is likely that the measured increases in  $\alpha$ -MyHC and decreases in  $\beta$ -MyHC gene expression were accompanied by changes in protein expression. Thus the movement toward normal in MyHC isoform expression is a molecular change that could causally relate to improved function, or at a minimum is a molecular marker of improved systolic function.

Similarly, the decrease in total MyHC ( $\alpha$ -MyHC +  $\beta$ -MyHC) gene expression with increasing LVEF (and decreasing end diastolic volume)<sup>22,40</sup> is a molecular measure of reverse remodeling, since MyHC is a major structural protein of the myocardial contractile element.<sup>48</sup> As a molecular index of reverse-remodeling changes in total MyHC or  $\beta$ -MyHC appeared to be superior to the indirect hypertrophic marker ANP, since total or  $\beta$ -MyHC but not ANP expression exhibited an incremental decline as remodeling progressively decreased. ANP gene expression decreased or tended to decrease in all LV function/diastolic volume change groups, indicating that this molecular phenotypic marker was responding to the general treatment of chronic heart failure.

There were also no changes in  $\beta_1$ -adrenergic receptor gene or protein expression or in SR  $\text{Ca}^{2+}$  ATPase gene expression that were systematically associated with changes in ventricular

function. Total  $\beta$ - and  $\beta_1$ -adrenergic receptor protein density increased or tended to increase in all 3 LV function change groups. This suggests that changes in these molecular markers of myocardial dysfunction can change with heart failure medical treatment, unrelated to ventricular function improvement or reversal of remodeling.

In summary, data presented in this study indicate that in subjects with IDC changes in MyHC isogene expression measured on septal endomyocardial biopsy are closely associated with improvements in left ventricular function and reversal of remodeling. These associations are specific for MyHC isoform expression, and are not observed for several other molecular changes previously shown to be present in the failing human heart. This may mean that in human dilated cardiomyopathy MyHC isoform dysregulation is an important molecular mechanism responsible for myocardial failure, or that  $\alpha$ -MyHC/ $\beta$ -MyHC gene expression is simply a relatively specific marker of myocardial dysfunction and pathologic remodeling. Future studies designed to evaluate the phenotypic and clinical consequences of directly normalizing MyHC isoform gene and protein expression will be required to determine which of these two possible interpretations is correct.

**Acknowledgments.** This investigation was sponsored by NIH Grant 1R01 HL48013 and by Glaxo SmithKline Pharmaceutical Company. The authors are grateful to Laurel Hunter for assistance in manuscript and figure preparation.

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## TABLES

**Table 1. Patient demographics (n = 45)**

Parameter	Value, $\pm$ SD
Age (Years)	54 $\pm$ 10.5
Gender (F/M)	20/25
LVEF (EF Units)	21.4 $\pm$ 8.2
RVEF (EF Units)	33.0 $\pm$ 10.6
Peak VO <sub>2</sub> (ml/kg/min)	16.0 $\pm$ 4.6
NYHA Class (II/III/IV)	9/35/1
Heart rate (beats/min)*	87.1 $\pm$ 17.8
Mean arterial pressure (mm Hg)*	85.7 $\pm$ 12.8
Right atrial mean pressure (mm Hg)	5.9 $\pm$ 4.8
Pulmonary artery mean pressure (mm Hg)	25.5 $\pm$ 9.4
Pulmonary wedge mean pressure (mm Hg)	13.6 $\pm$ 7.5
Cardiac index (L/min/m <sup>2</sup> )	2.46 $\pm$ 0.59
Stroke volume index (ml/beat/m <sup>2</sup> )	29.2 $\pm$ 9.1
LV stroke work index (g-m/m <sup>2</sup> )	29.5 $\pm$ 12.2
Coronary sinus norepinephrine (pg/ml)	1010 $\pm$ 680

\* measured at the time of catheterization

**Table 2. Baseline gene expression (molecules mRNA x 10<sup>5</sup>/μg total RNA or %) or β-adrenergic receptor density (fmol/mg protein or %)**

Parameter	Value, ± SD	
Gene Expression (molecules mRNA x 10 <sup>5</sup> /μg total RNA)	Nonfailing Controls n = 8	IDC, Failing n = 45
β <sub>1</sub> -adrenergic receptor (β <sub>1</sub> AR)	3.05 ± 2.05	1.67 ± 1.58*
β <sub>2</sub> -adrenergic receptor (β <sub>2</sub> AR)	2.65 ± 1.51	2.60 ± 1.81
Atrial natriuretic peptide (ANP)	34.0 ± 32.7	81.5 ± 55.1*
SR Ca <sup>2+</sup> ATPase-2a (SRCA)	82.8 ± 26.7	60.4 ± 23.4*
α-myosin heavy chain (α-MyHC)	37.1 ± 19.2	10.9 ± 6.7*
β-myosin heavy chain (β-MyHC)	114 ± 33	162 ± 66 <sup>#</sup>
Total myosin heavy chain	148 ± 39	172 ± 69
% α-MyHC	23.1 ± 8.1	6.5 ± 3.5*
% β-MyHC	76.9 ± 8.1	93.4 ± 3.5*
β-Adrenergic Receptor Protein (fmol/mg)	n = 8	n = 36
Total β-adrenergic receptor density	74.5 ± 34.6	48.7 ± 24.5*
β <sub>1</sub> -adrenergic receptor density	57.7 ± 29.4	29.5 ± 20.4*
β <sub>2</sub> -adrenergic receptor density	16.8 ± 10.3	19.3 ± 12.9
% β <sub>1</sub> -adrenergic receptors	77.0 ± 11.5	58.7 ± 21.4*
% β <sub>2</sub> -adrenergic receptors	23.0 ± 11.5	41.3 ± 21.4*
General Demographics	n = 8	n = 45

Age	49.1 ± 4.6	54.1 ± 10.5
Gender (F/M)	5/3	20/25
LVEF (EF Units)	58.9 ± 9.6	21.4 ± 8.2*
RVEF (EF Units)	47.6 ± 6.2	33.0 ± 10.6*

\*, p < .05 vs. nonfailing; #, p < .10 vs. nonfailing

**Table 3. Contingency Table Analysis of LV and RV Function Change Groups by Treatment, n and (%)**

Treatment Group	Decline/no change		Improvement		Marked Improvement	
	LV	RV	LV	RV	LV	RV
Placebo (n = 13)	9 (69)	6 (46)	2 (15)	4 (31)	2 (15)	3 (23)
Metoprolol (n = 14)	2 (14)	4 (29)	7 (50)	5 (36)	5 (36)	5 (36)
Carvedilol (n = 18)	4 (22)	5 (28)	6 (33)	6 (33)	8 (44)	7 (39)

Contingency Table Analysis:  $p = .021$  for LVEF;  $p = \text{ns}$  for RVEF

**Table 4. Changes in LVEF, RVEF, hemodynamics, coronary sinus norepinephrine and peak V<sub>O</sub><sub>2</sub> in the 3 LV Function Change groups (15 subjects in each group,  $\pm$  SEM)**

Parameter and units	Decline/no change	Improved	Marked Improvement
LVEF (EF Units)	$-2.2 \pm 1.1^{\S}$	$12.2 \pm 0.9^{*,\dagger}$	$25.5 \pm 1.8^{*,\#,\dagger}$
RVEF (EF Units)	$-0.2 \pm 1.9$	$10.2 \pm 2.3^{*,\dagger}$	$13.5 \pm 3.1^{*,\dagger}$
Heart rate (BPM)	$-14.2 \pm 5.5^{\dagger}$	$-15.4 \pm 3.7^{\dagger}$	$-19.9 \pm 4.8^{\dagger}$
Mean arterial pressure (mm Hg)	$-3.8 \pm 3.8$	$-2.1 \pm 4.7$	$-1.5 \pm 4.1$
Right atrial mean pressure (mm Hg)	$0.2 \pm 1.2$	$-0.1 \pm 1.2$	$-2.2 \pm 1.3$
Pulmonary artery mean pressure (mm Hg)	$-2.1 \pm 2.1$	$0.3 \pm 2.0$	$-9.9 \pm 2.1^{*,\#,\dagger}$
Pulmonary wedge mean pressure (mm Hg)	$-1.3 \pm 1.7$	$-0.2 \pm 1.8$	$-8.4 \pm 2.3^{\#,\dagger}$
Cardiac index (L/min/m <sup>2</sup> )	$0.02 \pm 0.19$	$-0.14 \pm 0.17$	$0.26 \pm 0.20$
Stroke volume index (ml/beat/m <sup>2</sup> )	$7.5 \pm 3.1^{\dagger}$	$5.4 \pm 2.3^{\dagger}$	$11.1 \pm 3.6^{\dagger}$
LV stroke work index (g-m/m <sup>2</sup> )	$6.1 \pm 2.8^{\S}$	$3.1 \pm 2.5$	$13.9 \pm 4.2^{\dagger}$
Coronary sinus norepinephrine (pg/ml)	$-258 \pm 168$	$-145 \pm 172$	$-206 \pm 209$
Peak V <sub>O</sub> <sub>2</sub> (ml/kg/min)	$1.0 \pm 1.5$	$0.8 \pm 1.4$	$1.3 \pm 0.9$

\*, p < .05 vs. Decline/no change; #, p < .05 vs. Improved; †, p < .05 within Group; §, p < .10

within Group

## FIGURE LEGENDS

1. Treatment protocol illustrating the three-way randomization between placebo, carvedilol and metoprolol. After 45 subjects were randomized the placebo arm was stopped for ethical reasons, and randomization between carvedilol and metoprolol continued. In all, 45 of the 49 subjects who completed this protocol had complete gene expression and ventricular function measurements at baseline and at six months.
2. Changes in radionuclide LVEF and RVEF in 3 "LV function change groups" constructed by arranging the change in LVEF in order and subdividing by the lowest 15 ("Decline/no change(NC)", middle 15 ("Improved") and highest 15 ("Marked improvement") in terms of degree of change.
3. Changes in  $\beta_1$ - and  $\beta_2$ -adrenergic receptor (AR) mRNA abundances in the 3 LV function change groups. P value refers to ANOVA.
4. Changes in atrial natriuretic peptide (ANP) and SR  $\text{Ca}^{2+}$  ATPase (SRCA) mRNA abundances in the 3 LV function change groups. Note y axis is logarithmic. P value refers to ANOVA.
5. Changes in  $\alpha$ - and  $\beta$ -myosin heavy chain (MyHC) mRNA abundances in the 3 LV function change groups. Note y axis is logarithmic. P value refers to ANOVA.

6. Changes in total ( $\alpha$ - plus  $\beta$ -) myosin heavy chain (MyHC) mRNA abundance and in %  $\alpha$ -MyHC/total MyHC in the 3 LV function change groups. Note y axis is logarithmic for total MyHC. P value refers to ANOVA.
  
7. Changes in  $\beta_1$ - and  $\beta_2$ -adrenergic receptor (AR) protein in the 3 LV function change groups. P value refers to ANOVA.

## FIGURES

Figure 1:

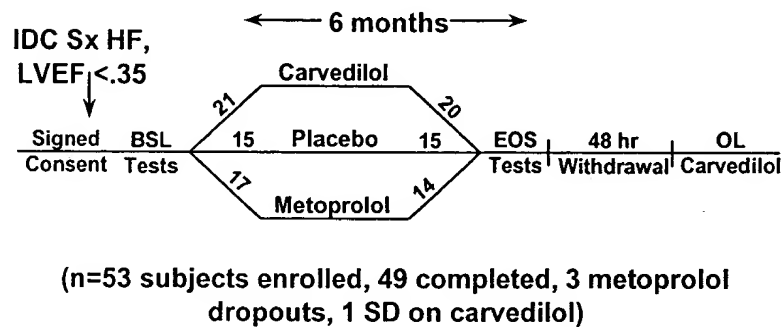


Figure 2:

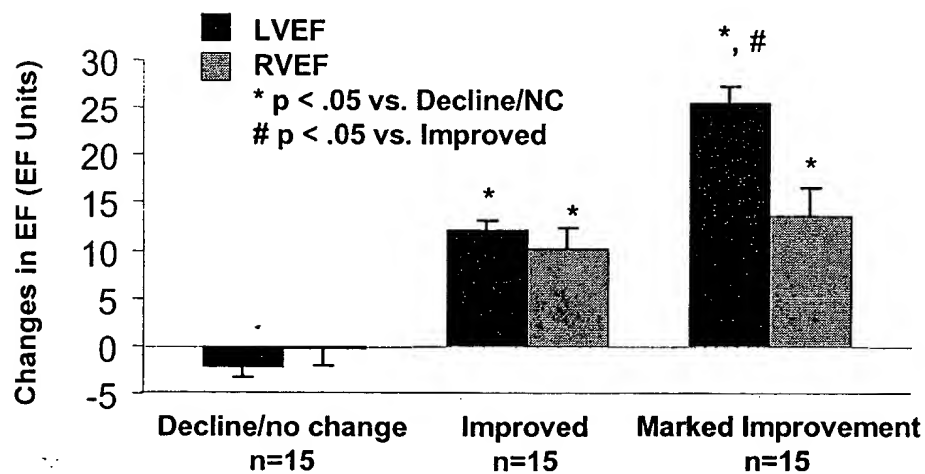


Figure 3:

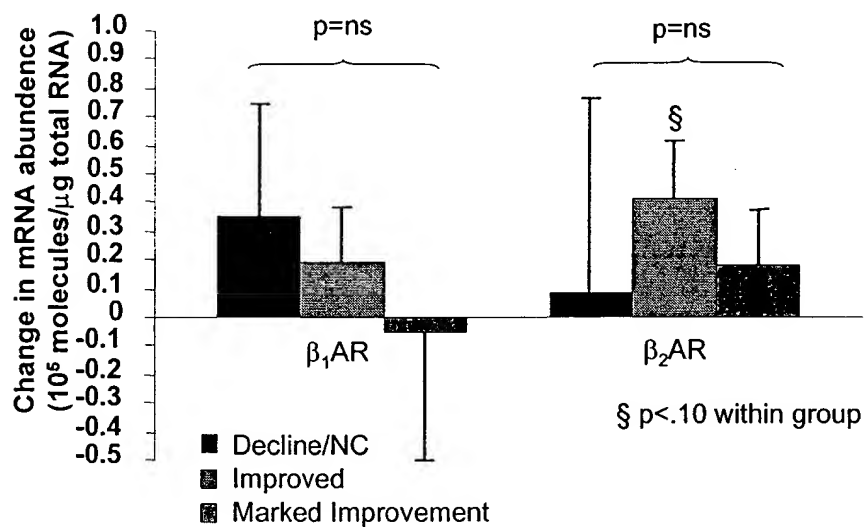


Figure 4:

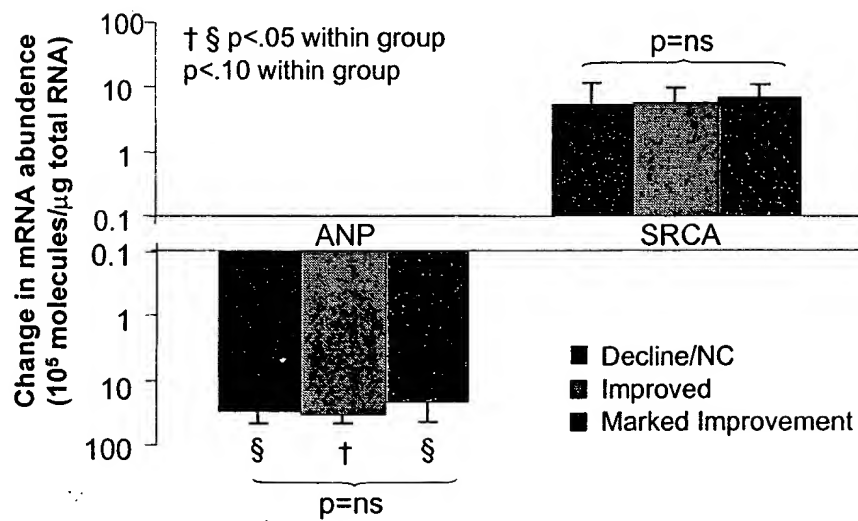


Figure 5:

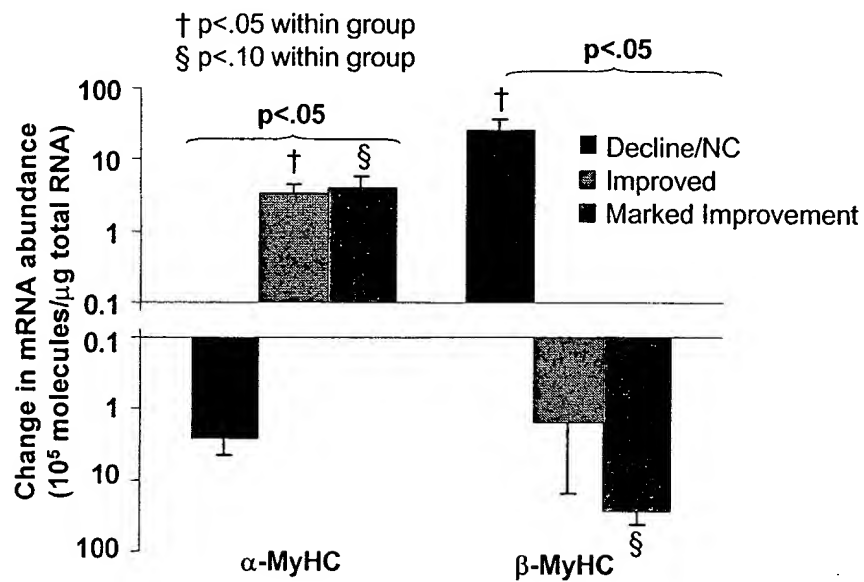


Figure 6:

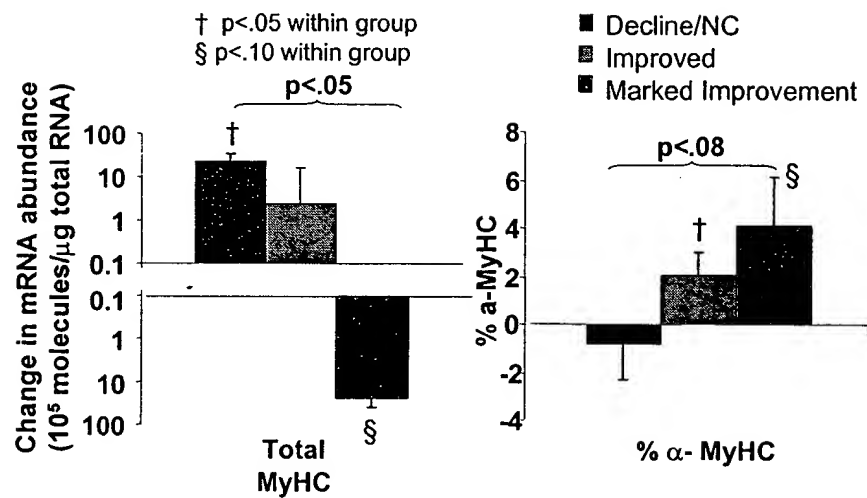
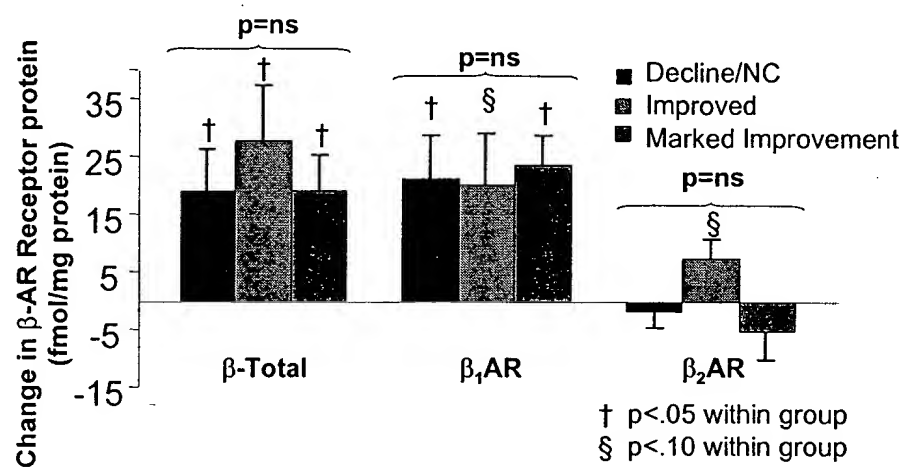


Figure 7:



## Figure Legend

1. Trial Design: HL 48013 - *UCHSC (Lowes, Bristow) UUHSC (Gilbert)*
2. Changes in LVEF and RVEF in the 3 LV function groups
3. Changes in  $\beta_1$ - and  $\beta_2$ -adrenergic receptor gene expression in the 3 LV function change groups
4. Changes in atrial natriuretic peptide (ANP) and SR  $\text{Ca}^{2+}$  ATPase (SRCA) gene expression in the 3 LV function change groups
5. Changes in  $\alpha$ - and  $\beta$ -myosin heavy chain (MyHC) gene expression in the 3 LV function change groups
6. Changes in %  $\alpha$ -MyHC and Total MyHC Gene Expression in the 3 LV function change groups
7. Changes in  $\beta_1$ - and  $\beta_2$ -adrenergic receptor protein expression in the 3 LV function change groups